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Somatic Histone H1 from Blastomere Nuclei in Oocyte Cytoplasm: A Potential Marker of Nuclear Reprogramming

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Nuclei of differentiated cells can acquire totipotency following transfer into the cytoplasm of oocytes. While the molecular basis of this nuclear reprogramming remains unknown, the developmental potential of nuclear-transfer embryos is influenced by the cell-cycle stage of both donor and recipient. As somatic H1 becomes immunologically undetectable on bovine embryonic nuclei following transfer into ooplasm and reappears during development of the reconstructed embryo, suggesting that it may act as a marker of nuclear reprogramming, we investigated the link between cell-cycle state and depletion of immunoreactive H1 following nuclear transplantation. Blastomere nuclei at M-, G1-, or G2-phase were introduced into ooplasts at metaphase II, telophase II, or interphase, and the reconstructed embryos were processed for immunofluorescent detection of somatic histone H1. Immunoreactivity was lost more quickly from donor nuclei at metaphase than at G1 or G2. Regardless of the stage of the donor nucleus, immunoreactivity was lost most rapidly when the recipient cytoplast was at metaphase and most slowly when the recipient was at interphase. When the recipient oocyte was not enucleated, however, immunoreactive H1 remained in the donor nucleus. The phosphorylation inhibitors 6-DMAP, roscovitine, and H89 inhibited the depletion of immunoreactive H1 from G2, but not G1, donor nuclei. In addition, immunoreactive H1 was depleted from mouse blastomere nuclei following transfer into bovine oocytes. Finally, expression of the developmentally regulated gene, eIF-1A, but not of Gapdh, was extinguished in metaphase recipients but not in interphase recipients. These results indicate that evolutionarily conserved cell-cycle-regulated activities, nuclear elements, and phosphorylation-linked events participate in the depletion of immunoreactive histone H1 from blastomere nuclei transferred in oocyte cytoplasm and that this is linked to changes in gene expression in the transferred nucleus. **© 2001 Academic Press**

Key Words: **somatic histone H1; chromatin remodeling; nuclear transfer; mouse.**

INTRODUCTION

Elucidating the molecular mechanisms involved in the resetting of the developmental program after the transplantation of nuclei into host oocytes is an intriguing biological problem and represents a necessary step for the application of nuclear transfer technologies. Although it is now clear that nuclei derived from differentiated cells from adult mammals can support development to term (Wilmut *et al.*, 1997; Kato *et al.*, 1998; Wakayama *et al.*, 1998), the molecular modifications accompanying the reprogramming of the transplanted nuclei remain almost entirely unknown. Initial studies using amphibians identified several morphological and functional modifications of somatic nuclei after transplantation into egg cytoplasm. These changes included nuclear decondensation, induction of DNA synthesis, influx of cytoplasmic proteins into the nuclei, exchange of proteins between nuclei and cytoplasm, cessation of RNA synthesis by the transplanted nuclei, and repression and reactivation at the normal stage of embryonic development of specific genes (reviewed by Gurdon, 1986).

Mammalian somatic cell nuclei transplanted into oocyte cytoplasts also undergo modifications that are suggestive of reprogramming, including nuclear swelling (Czolowska *et*

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al., 1984), in nuclear lamins (Prather *et al.,* 1991) and changes in the patterns of RNA (Dyban *et al.,* 1988) and protein synthesis (Howlett *et al.,* 1987; Latham *et al.,* 1991a). Moreover, early embryonic nuclei transferred to enucleated one-cell embryos are able to direct synthesis of a set of proteins, termed the transcription-requiring complex, that are markers of embryonic genome activation (Latham *et al.,* 1991b). Although these studies imply that oocyte cytoplasts are able to reprogram foreign nuclei, other studies have identified abnormal patterns of nuclear activity in such reconstructed embryos. A quantitative study of the pattern of protein synthesis directed by eight-cell stage nuclei transferred to one-cell cytoplasm revealed significant alterations from the normal pattern of protein synthesis, implying that they are unable to recapitulate completely the normal progression of changes in protein synthesis that occur during early cleavage (Latham *et al.,* 1994). Moreover, early one-cell host embryos were unable to restore the activity of repressed promoters in transplanted two-cell stage nuclei (Henery *et al.,* 1995). Overall, notwithstanding the progress in characterizing the functional capacity of transplanted nuclei, the molecular basis of reprogramming remains largely unknown.

Numerous studies employing different mammalian species have demonstrated a key effect of the cell-cycle stage of both the donor nucleus and the host cytoplasm on the developmental potential of reconstructed embryos (Smith *et al.,* 1988; Otaegui *et al.,* 1994). Moreover, the remodeling of somatic (Szöllösi *et al.,* 1988) and embryonic (Collas and Robl, 1991) nuclei, as measured by the swelling rate, was more extensive with metaphase than interphase cytoplasts. Chromatin replicative and transcriptional activity of transplanted nuclei also was modified faster and more effectively when using metaphase than interphase cytoplasts (Barnes *et al.,* 1993). Together, these results indicate that the cell-cycle stage influences the reprogramming capabilities of reconstructed embryos and, therefore, may act directly on the remodeling the chromatin of transplanted nuclei.

It has long been speculated that the developmental modifications occurring to the chromatin during cellular differentiation affect the capability of transplanted nuclei to regain totipotency. This idea is supported by the poor development potential of mouse nuclei derived from more advanced embryonic stages compared with those obtained before or after a few cleavages (McGrath and Solter, 1984). Moreover, as evidenced in other species, nuclei derived from early stage embryos show higher developmental potential (Campbell *et al.,* 1994) than those derived from somatic cells (Campbell *et al.,* 1996; Wilmut *et al.,* 1997). A plausible explanation for this developmental restriction may involve structural modifications to the chromatin during cellular differentiation, which may interfere with a complete remodeling of transplanted nuclei.

Linker histone H1 may be actively involved in regulating gene expression during early embryonic development (reviewed by Clarke *et al.,* 1998). Oocytes and early embryos of *Xenopus* lack the somatic form of histone H1, which first becomes detectable on chromatin at about the stage of the major transcriptional activation of the embryonic genome (Ohsumi and Katagiri, 1991). Experimental acceleration or delay of the timing of the switch from oocyte-type to somatic-type H1 correspondingly alters the time when mesoderm-inducing genes can be activated (Steinbach *et al.,* 1997). In mice and cattle, somatic H1 cannot be detected by immunocytochemical means on the chromatin until near the time of the major transcriptional activation of the embryonic genome in each species (Clarke *et al.,* 1992; Smith *et al.,* 1995). Following nuclear transplantation in the cow, histone H1 immunoreactivity is lost from the donor nucleus and reappears according to the normal schedule in the reconstructed embryos (Bordignon *et al.,* 1999). These results suggest that linker histones may be involved in the early genomic function in mammals and can also serve as a molecular marker to study the remodeling of chromatin structure and function after nuclear transplantation.

In the studies reported here, we investigated whether nuclear transplantation in mice was accompanied by changes in the immunoreactivity of histone H1. We further examined whether these changes were regulated by the cell-cycle stage of the donor nucleus and host cytoplasm, by host nuclear factors, and by protein phosphorylation. Finally, we verified whether the expression of developmentally regulated genes in reconstructed embryos was affected by the cell-cycle stage of the host cytoplasm at nuclear transfer.

MATERIALS AND METHODS

Collection and Culture of Mouse Oocytes and Embryos

Hybrid F1 females (C57Bl/6 × C3H; Charles River Canada, St.-Constant, Quebec, Canada), were superovulated by intraperitoneal injection of 5 IU of pregnant mare serum gonadotrophin (PMSG; Folligon; Ayerst, Montreal, Canada) and 5 IU of human chorionic gonadotrophin (hCG; Ayerst) given 46–48 h apart. Oocytes used as host cytoplasms were collected from the ampullae of the oviducts at 15–18 h after hCG injection. Cumulus cells were removed by treatment with 0.1% (w/v) hyaluronidase (Sigma, St. Louis, MO) in Hepes buffered CZB medium (Chatot *et al.,* 1989). The oocytes then were placed in 50-μl droplets of glucose-free, bicarbonate-buffered CZB medium under mineral oil (Sigma) at 37°C in a humidified atmosphere of 5% CO_2 in air until activation or nuclear transfer. Host oocytes were parthenogenetically activated at 20–24 h after hCG injection by exposure to 7% ethanol (v/v) in Hepes-buffered CZB medium for 5 min, washed three times, and cultured in bicarbonate-buffered CZB medium to allow the extrusion of the second polar body and pronuclear formation. To produce nuclear-donor embryos, superovulated females were paired with CD-1 males (Charles River) and inspected the following morning for copulation plug. Embryos were flushed from the oviducts at 56 h post-hCG and good-quality four-cell stage embryos were selected.

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Synchronization of Blastomeres

Four-cell embryos were cultured in the presence of 0.33 μM methyl(5-[2-thienylcarbonyl]-1*H*-benzimidazol-2-yl)carbamate (nocodazole; Sigma) in CZB medium for 6–10 h to synchronize cells at metaphase (Samaké and Smith, 1996). After nocodazole treatment, embryos were used either immediately (metaphase stage) or washed several times and cultured for 3 (G1/early S stage) or 8 h (late S/G2 stage). As the cleavage of arrested blastomeres occurred 0.5–1 h after release from nocodazole, nuclei were thus used at 2–2.5 or 7–7.5 h postcleavage. Before micromanipulation, embryos were exposed to a 0.1% pronase solution for 3 min to remove the zona pellucida, followed by disaggregation of blastomeres using a fine bore pipette.

Nuclear Transfer

All micromanipulations were performed in Hepes-buffered CZB medium containing 1 μg ml^{-1} cytochalasin D (Sigma) and 0.1 μg ml^{-1} of nocodazole. Before enucleation, metaphase host cytoplasms were incubated for 5 min with the DNA vital stain bisbenzimide (Hoechst 33342, 2 μg ml^{-1}, Sigma) and a small volume of the cytoplasm surrounding the metaphase plate was removed. Cytoplasmic fragments were exposed to ultraviolet irradiation to verify enucleation. A blastomere was immediately injected into the perivitelline space of the enucleated recipient and the membranes were fused by electrostimulation consisting of a 1.5-kV/cm electric pulse for 60 μs in a 0.3 M mannitol solution containing 0.1 mM $MgSO_4$ and 0.05 mM $CaCl_2$. After electrofusion, reconstructed oocytes were washed and cultured in bicarbonate-buffered CZB medium until fixation. Telophase host cytoplasts were selected by the presence of the second polar body at 1.5 h postactivation and stained with Hoechst dye. The second polar body and a small portion of the surrounding cytoplasm were removed and exposed to ultraviolet irradiation to confirm enucleation. Electrofusion with donor blastomeres was performed 3 h postactivation. Interphase host cytoplasms were selected by the presence of a pronucleus at 6.5 h postactivation, enucleated without chromatin staining, and used for fusion at 8 h postactivation. When donor nuclei were obtained at metaphase before cleavage to eight-cells, only a karyoplast consisting of the chromatin and 50% of the blastomere cytoplasm were transferred to the host cytoplasm. The karyoplast was obtained by staining the nocodazole-arrested four-cell blastomeres and exposure to UV light for a few seconds to ascertain the presence of chromatin. When the donors were eight-cell interphase nuclei at G1/early S and late S/early G2, however, the entire blastomere was transferred.

Bovine Oocyte Source and Manipulation

Bovine oocytes were obtained by follicular aspiration from slaughterhouse-derived ovaries. Follicles with diameters between 2 and 8 mm were punctured with a 19-gauge needle and cumulus–oocyte complexes (COCs) with several layers of cumulus cells and homogeneous oocyte cytoplasm were washed in Hepes-buffered tissue culture medium (TCM-199; Gibco BRL, Burlington, Ontario, Canada) supplemented with 10% (v/v) fetal calf serum (FCS, Gibco). Groups of 20 COCs were placed in 100 μl of bicarbonate-buffered TCM-199 supplemented with 10% FCS, 50 μg ml^{-1} LH (Ayerst, London, Ontario, Canada), 0.5 μg ml^{-1} FSH (Follitropin-V; Vetrepharm, Montreal, Quebec, Canada), 1 μg ml^{-1} 17β-estradiol (Sigma), 22 μg ml^{-1} pyruvate (Sigma), and 50 μg ml^{-1} gentamicin (Sigma).

Oocytes were denuded of cumulus cells after 24 h of maturation, selected for the presence of the first polar body, and micromanipulated in PBS containing 7.5 μg ml^{-1} cytochalasin B (Sigma). Metaphase II oocytes were enucleated by removing approximately 30% of the cytoplasm adjacent to the first polar body, placed in medium containing 5 μg ml^{-1} Hoechst 33342 for 10 min, and exposed briefly to ultraviolet irradiation to verify by the absence of chromatin that enucleation was complete. Telophase and interphase oocytes were exposed to an activation stimulus with ionomycin (Sigma) and returned to culture to allow for extrusion of the second polar body. After 2 h from exposure to ionomycin, activated oocytes were enucleated by removing a small portion of the cytoplasm adjacent to the second polar body (Bordignon and Smith, 1998). Oocytes to be reconstructed at interphase were returned to the culture for an additional 5 h before nuclear transfer and electrofusion.

Immunocytochemistry

Groups of nuclear-transfer reconstructed oocytes were fixed in 10% formalin (Sigma) for 20 min, washed, and stored at 4°C in 0.9% saline containing 0.1% Tween 20. To detect somatic histone H1, fixed oocytes were incubated in a blocking solution (PBS, 3% BSA, 0.5% Triton X-100) for 1 h at room temperature, transferred to anti-histone H1 antibody (raised in rabbit using histone H1 from rat thymus and affinity purified (Bustin and Stollar, 1973; Sluyser and Bustin, 1974; Clarke *et al.*, 1992) diluted in 1:50 in blocking solution, and then incubated overnight at 4°C. This antibody has previously been shown to recognize somatic H1 subtypes but not H1 subtypes present in mouse and bovine oocytes and early embryos (Clarke *et al.*, 1992; Smith *et al.*, 1995). The cells were then washed twice in blocking solution, incubated in fluorescein-conjugated goat (anti-rabbit) IgG diluted 1:100 in blocking solution for 1 h at room temperature, and washed as above. Specimens were mounted on slides in a mounting medium containing Mowiol (Hoechst), the DNA stain DAPI (1 μg ml^{-1}, Sigma), and the anti-fading agent, Dabco (Sigma). Oocytes and embryos were examined using standard epifluorescence optics.

Drugs Used to Inhibit Cellular Activities

Reconstructed oocytes were exposed for a period of 4 h to inhibitors of DNA replication (aphidicolin, 100 μg ml^{-1}; Boehringer), protein synthesis (cycloheximide, 10 μg ml^{-1}; Sigma), protein phosphatases (okadaic acid, 1μM; Sigma), protein phosphorylation (6-DMAP, 3 mM; Sigma), and specific inhibitors of cdc2 kinase (roscovitine, 100 μM; Biomol, Plymouth Meeting, PA), protein kinase A (H-89, 30 μM, Biomol), protein kinase C (GF 109203X, 1 μM; Biomol), and MEK (PD-98059, 100 μM; Biomol), before being fixed and processed for immunofluorescence.

Gene Expression in Reconstructed Embryos

The relative abundance of eIF-1A and Gapdh mRNA transcripts in one- and two-cell stages embryos reconstructed with MII and interphase host cytoplasts was measured by semiquantitative reverse transcription–polymerase chain reaction (RT-PCR). Nonmanipulated embryos at the two- and eight-cell stage, MII oocytes, and oocytes at 6 and 14 h postactivation were included as controls. Activated oocytes served as controls for one-cell embryos reconstructed with host cytoplasts at MII and interphase, respectively. Extraction of mRNA was performed with the RNeasy mini kits (Qiagen Inc., Mississauga, Ontario, Canada), from a pool of 5 to 15

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oocytes or embryos per extraction. Prior to mRNA extraction 0.25 pg of rabbit α-globin mRNA (Gibco BRL) was added as an internal control. Extracted mRNA products were resuspended in 20 μl of water and reversed transcribed accordingly (Omniscript RT kit; Qiagen). After reverse transcription, 3 μl of each reaction was used for amplifying α-globin and a volume equivalent to 1 or 1.5 oocytes or embryos was used for the amplification of eIF-1A and Gapdh, respectively. Primers were designed using known sequences, α-globin (5′–3′, 5′-GCAGCCACGGTGGCGAGTAT-3′; and 3′–5′, 5′-GTGGGACAGGAGCTTGAAAT-3′) (Temeles *et al.*, 1994), eIF-1A (5′–3′, 5′-ATTGCTGGGAAATGGACGGTTGGA-3′; and 3′–5′, 5′-GTTCTCCATAGGCCTTCAGACTTC-3′) (Davis and Schultz, 1998), and Gapdh (5′–3′, 5′-CAGCCTCGTCCCGTAGACAAAATGG-3′; and 3′–5′, 5′-TTCTGGGTGGCAGTGATGGCATGGA-3′) (Eversole-Cire *et al.*, 1995). PCR amplification consisted of 34, 30, and 28 cycles of a transcript-specific annealing at 56°C/60 s, 56°C/45 s, and 54°C/45 s and extension at 72°C for 20, 45, and 45 seconds for α-globin, eIF-1A, and Gapdh, respectively. A constant volume of the PCR product was electrophoresed, stained with ethidium bromide, and quantified by densitometry. At least three replicates of each treatment were performed and the amount of amplified mRNA transcripts for eIF-1A and Gapdh were expressed in proportion to α-globin.

Statistical Analysis

Frequencies of somatic H1 staining among groups of nuclear-transfer embryos were analyzed by χ^2.

RESULTS

Loss of Immunoreactive H1 from Mouse Blastomere Nuclei Exposed to Cytoplasm of Activated Mouse Oocytes

In the mouse and the cow, somatic H1 is immunologically undetectable on embryonic nuclei during early cleavage stages, becoming detectable at the 2- to 4-cell stage in mice and the 8- to 16-cell stage in the cow (Clarke *et al.*, 1992, 1998; Smith *et al.*, 1995). When H1-immunoreactive nuclei of morulae are transferred into oocytes or 1-cell embryos, immunoreactivity is lost within 6 h (Bordignon *et al.*, 1999), suggesting that oocyte cytoplasm possesses an activity that causes loss of immunoreactive H1 from blastomere nuclei. To begin to identify the properties of this activity, we first tested whether the same loss of immunoreactive H1 occurred following nuclear transfer into mouse oocytes. Nuclei at G1/S obtained from 8-cell embryos were fused to activated mouse oocytes from which the chromosomes had been removed, and the recipients were fixed at different times following fusion.

Immunoreactive H1 was easily detected in nuclei of recipients (obtained at telophase II) fixed shortly after fusion (Fig. 1). During the next several hours, however, it became undetectable. Although the nuclei also enlarged during residence in the ooplast, there was no strict correlation between nuclear size and H1 immunoreactivity (data not shown). When the recipients were incubated for 16 and 20 h postfusion, which corresponds temporally to the early two-cell stage, immunoreactive H1 reappeared weakly in most transferred nuclei. Thus, the fate of immunoreactive H1 following nuclear transfer in the mouse was similar to that in the cow—it was lost following nuclear transfer, albeit more rapidly in the mouse than in the cow, and reappeared during subsequent development.

Effect of Cell-Cycle Stage of Donor Nucleus and Host Cytoplasm on Loss of Immunoreactive H1

As discussed above, the cell-cycle state of both donor nucleus and recipient cytoplasm influence the developmental potential of nuclear-transfer embryos. To test whether the cell cycle also regulated loss of immunoreactive H1, the following experiments were performed. Donor nuclei at different stages of the cell cycle were obtained by treating four-cell-stage embryos with nocodazole to arrest them at metaphase and then removing the nocodazole and collecting blastomeres after 0 h (M-phase), 3 h (G1-phase), or 8 h (G2-phase) of culture. Recipient oocytes were obtained at metaphase II or at 3 h (telophase II) or 8 h (interphase) after parthenogenetic activation. Host chromosomes or nuclei were removed about 1 h before fusion. Donor nuclei at each stage were fused to host oocytes at each stage, and samples were fixed at different times after fusion and processed for immunofluorescence.

First, we investigated the role of the donor nucleus (Table 1, Fig. 2). When M-phase blastomeres were transferred to telophase II ooplasm, staining was lost from donor nuclei by 2 h postfusion. When G1 or G2 blastomeres were used, however, staining was not lost until 4 h postfusion. Thus, in telophase II recipients, the timing of the loss of immunoreactive H1 was influenced by the cell cycle stage of the donor nucleus. Similarly, using interphase recipients, staining was in some cases lost from M-phase donor nuclei but only rarely from G1 or G2 donor nuclei. Using M-phase recipients, however, immunoreactive H1 was lost by 2 h regardless of the source of the nucleus. Thus, in telophase and interphase ooplasm, immunoreactive H1 is lost more rapidly from M-phase nuclei than from G1 or G2 nuclei.

Next, we investigated the role of the recipient ooplasm (Table 1, Fig. 2). When donor nuclei at M, G1, or G2 were transferred to metaphase II ooplasm, immunoreactive H1 was lost by 2 h postfusion. By contrast, when G1 or G2 nuclei were transferred to telophase II ooplasm, immunoreactive H1 was not lost until 4 h postfusion. Furthermore, when G1 or G2 nuclei were transferred to interphase ooplasm, immunoreactive H1 was not lost at any time after fusion. Thus, independently of the cell-cycle stage of the donor, H1 immunoreactivity was lost most rapidly following nuclear transfer in M-phase cytoplasm and most slowly in interphase cytoplasm. This suggests that the oocyte cytoplasmic activity that is responsible for loss of H1 immunoreactivity declines after activation.

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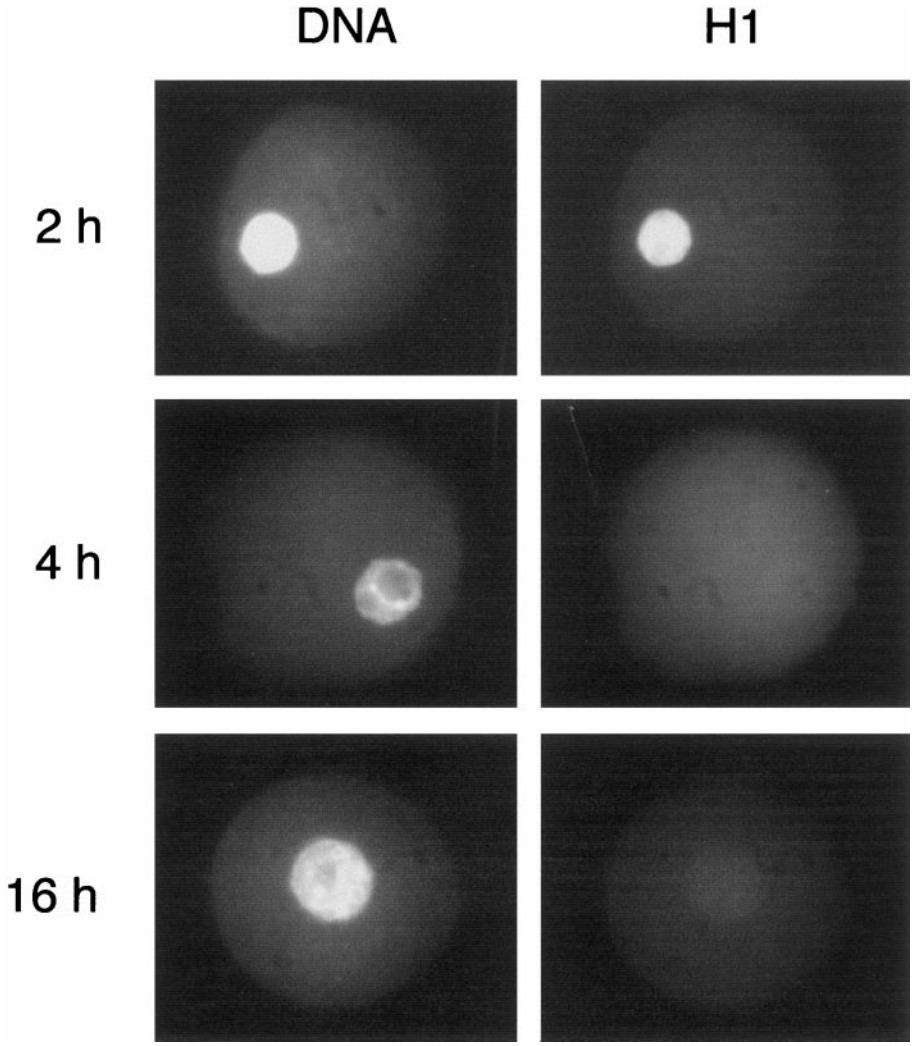

FIG. 1. Loss of immunoreactive histone H1 from blastomere nuclei transplanted into activated oocyte cytoplasm. Specimens shown are of nuclei obtained at G1/S (3 h postnocodazole), fused with host ooplasts at telophase II (3 h postactivation), and fixed at 2, 4, and 16 h postfusion. (Right) Immunostaining of histone H1; (left) DAPI-stained DNA. 400× magnification.

Role of Nuclear Components of Activated Oocytes in Loss of Immunoreactive H1

Two explanations could account for inability of interphase ooplasm to remove immunoreactive H1 from donor nuclei. First, activation of the oocyte might trigger a progressive decline in the activity. Hence, oocytes used at later times following activation would possess less activity. Second, the activity could depend on factors that accumulate in nuclei. As the host oocyte nuclei were removed only 1 h before fusion, these factors would have been removed from the recipients that were enucleated at interphase.

To distinguish between these possibilities, the following experiment was carried out using three groups of recipients. In the first, oocytes were enucleated 7 h postactivation and used for fusion at 8 h postactivation. In this case, which is the same protocol used for the data of Table 1, all nuclear components would be removed by the enucleation process. In the second group, the host oocytes were not enucleated and were used at 8 h postactivation. In this group, the soluble nuclear components would remain present but would be associated with the host nucleus. In the third group, the host oocyte chromosomes were removed at 1.5 to 3 h postactivation, and the ooplasts were returned to culture and used for fusion at 8 h postactivation. In this case, nonchromosomal nuclear components would remain in the host ooplast. Blastomere nuclei at G1 were fused to these recipients, and the reconstructed embryos were fixed at 2 and 4 h postfusion and processed for immunofluorescence.

As observed in the previous experiment, when the recipi-

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TABLE 1

Effect of Cell Cycle Stage of the Donor Nucleus and the Recipient Cytoplasm on the Timing of the Loss of Immunoreactive H1 Following Nuclear Transplantation

Stage of donor nucleus	Time fixed (h postfusion)	Cell-cycle stage of recipient ooplast at time of fusion[a]								
		Metaphase II			Telophase II (3 h)			Interphase (8 h)		
		n	+	−	*n*	+	−	*n*	+	−
M	2	9	0	9	11	1	10	16	12	4
	4	9	0	9	12	0	12	13	4	9
	16	9	5	4	12	12	0	13	5	8
G1/S	1	8	3	5	5	4	1	6	6	0
	2	9	1	8	10	6	4	7	7	0
	4	9	0	9	7	0	7	8	7	1
	8	5	0	5	5	0	5	8	8	0
	16	7	0	7	7	5	2	10	5	5
	20	4	4	0	7	7	0	6	4	2
S/G2	2	10	1	9	9	9	0	8	8	0
	4	9	0	9	9	1	8	10	10	0
	16	10	0	10	10	10	0	11	11	0

[a] (+) and (−), denote the presence and absence of somatic H1 staining of nuclei, respectively.

ent ooplasts had been enucleated at 7 h, most donor nuclei retained immunoreactive H1 at 2 h and 4 h postfusion (Fig. 3). Similarly, when the host oocyte nuclei were not removed, the donor nuclei retained immunoreactive H1 and 2 and 4 h postfusion. Figure 4 illustrates one such reconstructed oocyte, where the stained donor nucleus and unstained host nucleus are clearly distinguishable. By contrast, when the chromosomes had been removed from the recipient oocyte up to 3 h postactivation, leaving the nonchromosomal nuclear components in the remaining ooplasm, immunoreactive H1 was present in significantly fewer donor nuclei at both 2 and 4 h postfusion ($P < 0.05$). As all groups were used for fusion at the same time after activation, this difference is not due an activation-triggered decline in the activity that causes loss of immunoreactive H1 from donor nuclei. Rather, the results are consistent with the possibility that this activity depends on factors that accumulate in the nucleus of the activated oocyte.

Role of Phosphorylation in Loss of Immunoreactive H1 from Transplanted Nuclei

The loss of immunoreactive H1 from blastomere nuclei within as little as 2–4 h after transfer into activated ooplasm suggested that DNA replication and RNA synthesis were unlikely to play significant roles in this process. We examined whether protein phosphorylation, which has been implicated in the removal both of sperm-specific histones following fertilization and of somatic histones following nuclear transfer in amphibians (Green and Poccia, 1985; Leno *et al.*, 1996; Dimitrov and Wolffe, 1996), was linked to loss of immunoreactive H1 following nuclear transfer.

Blastomeres at G1 or G2 were fused to metaphase ooplasts, and the recipients were cultured in the presence of phosphorylation inhibitors for 4 h and then processed for immunofluorescence (Table 2). The general phosphorylation inhibitor, 6-DMAP, had no apparent effect on the loss immunoreactive H1 from donor G1 nuclei. In contrast, 6-DMAP prevented the loss of immunoreactive H1 in about half of the recipients of G2 nuclei. Furthermore, inhibitors of protein kinase A (H89, 14 fused cells examined) or of cdk kinases (roscovitine, 12 fused cells examined) each prevented the loss of immunoreactive H1 from G2 nuclei in all recipients. However, inhibitors of PKC and MAP kinase, and the phosphatase inhibitor okadaic acid, showed no effect (data not shown). These results suggest that a phosphorylation-dependent mechanism regulates the loss of immunoreactive H1 from G2 nuclei in activated oocyte cytoplasm.

Loss of Immunoreactive H1 from Mouse Blastomere Nuclei in Bovine Oocyte Cytoplasm

Previous work established that immunoreactive H1 is lost from bovine morula-stage nuclei transferred into bovine oocytes (Bordignon *et al.*, 1999). To examine whether the mechanisms underlying the loss of immunoreactive H1 are conserved among species, mouse eight-cell-stage blastomeres at G1 were fused to enucleated bovine oocytes at metaphase II, telophase II (3 h postactivation), or interphase (8 h postactivation). Recipients were fixed at regular intervals after fusion and processed for immunofluorescence.

As shown in Table 3, bovine cytoplasts at all three stages of the cell cycle were able to induce loss of immunoreactive H1 from the mouse nuclei. When the nuclei were trans-

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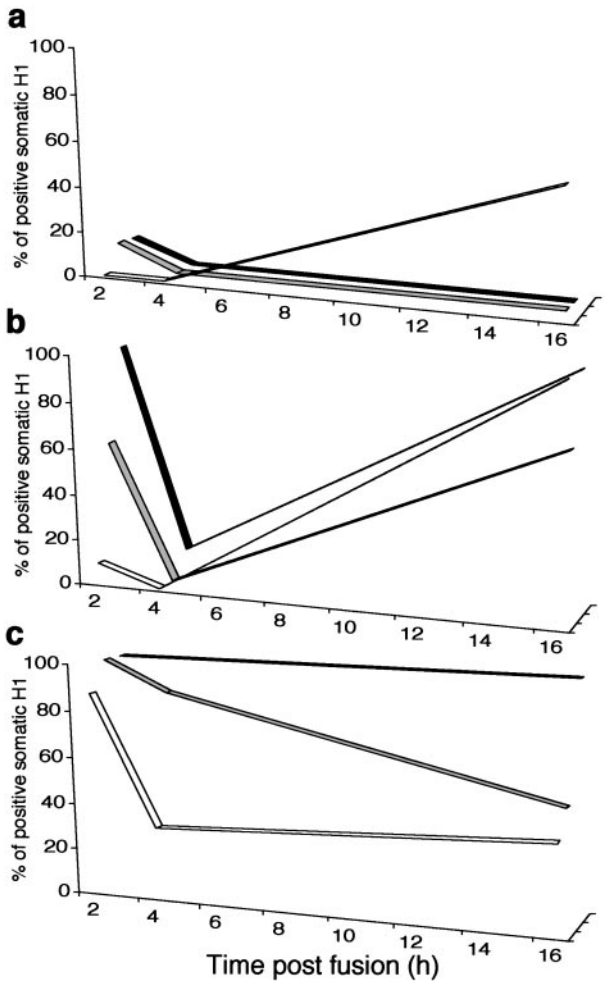

FIG. 2. Effect of cell-cycle stage of the donor nucleus and the recipient cytoplasm on loss of immunoreactive H1 following nuclear transplantation. Values represent the percentage of nuclei that contained detectable immunoreactive H1 at different times following transfer into ooplasts at (a) metaphase II, (b) telophase II, or (c) interphase. Nuclei were transferred at metaphase (white line), G1/early S (gray line), or late S/G2 (black line).

ferred to metaphase cytoplasts, immunoreactive H1 was lost by 1 h postfusion. When they were transferred to telophase and interphase cytoplasts, however, immunoreactive H1 was not lost until 2 and 4 h after fusion, respectively. Thus, as observed in intraspecific nuclear transfer, metaphase cytoplasm caused a more rapid loss of immunoreactive H1 in interspecific nuclear transfers. Moreover, by comparing Table 3 with Table 1, it may be seen that immunoreactive H1 was lost more rapidly from mouse nuclei exposed to bovine cytoplasts than from those exposed to mouse cytoplasts. This effect was observed regardless of the cell cycle stage of the recipient. Thus, the activity that mediates loss of H1 immunoreactivity from

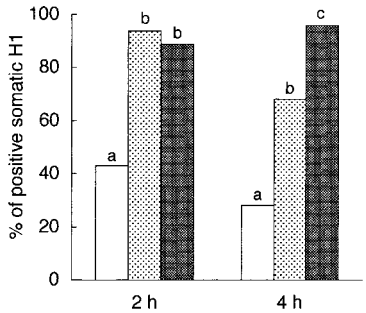

FIG. 3. Effect of host nuclear components on the loss of immunoreactive H1 following nuclear transfer. Bars indicate the fraction of eight-cell-stage nuclei that retained detectable immunoreactive H1 following transfer into interphase-stage cytoplasts that had been enucleated before pronuclear growth (white), after pronuclear growth (light gray), or not enucleated (dark gray).

blastomere nuclei apparently is stronger in bovine ooplasts than in mouse ooplasts.

Gene Expression in Reconstructed Embryos

In order to verify whether the cell-cycle stage of the cytoplast affects the transcriptional activity of the transplanted chromatin, the expression pattern of eIF-1A and Gapdh genes was determined in embryos reconstructed using MII and interphase host oocytes. Although the expression of the housekeeping gene Gapdh was not affected by the stage of the host cytoplasts, the developmentally regulated gene eIF-1A was expressed differently between embryos reconstructed using MII and interphase host oocytes (Fig. 5). At the one-cell stage, eIF-1A was expressed more abundantly in the embryos reconstructed using interphase hosts than using MII hosts. No difference in expression was observed between the MII reconstructed embryos

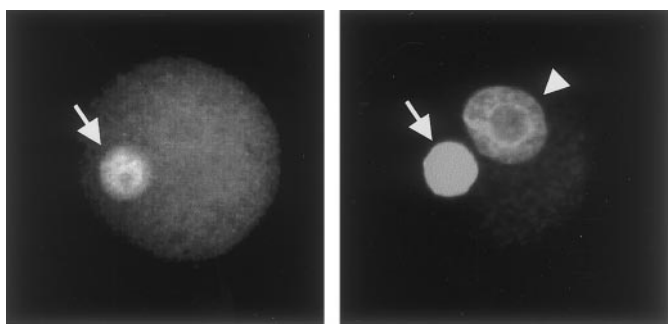

FIG. 4. Prolonged retention of immunoreactive H1 in eight-cell-stage nuclei transferred to nonenucleated host cytoplasm. Specimen was fixed 8 h postfusion. Both donor (arrow) and host (arrowhead) nuclei are visible by DAPI staining (right); H1 is detectable on donor but not host nucleus (left).

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TABLE 2

Effect of 6-DMAP on the Loss of Immunoreactive H1 from Blastomere Nuclei Following Transplantation into Activated Oocyte Cytoplasm

Cell-cycle stage of donor nucleus	Treatment	No. cases	Stained	Unstained
G1/early S	None	12	0	12
	6-DMAP	33	1	32
Late S/G2	None	13	0	13
	6-DMAP	29	21	8

and control nonmanipulated MII or activated oocytes. These results indicate that donor eight-cell nuclei remain transcriptionally active after fusion with interphase host cytoplasm. Moreover, as the amount of eIF-1A detected was substantially higher than would be expected from a single eight-cell blastomere, i.e., one-eighth of the message present in an entire eight-cell stage embryo, it appears that the eight-cell donor nuclei exposed to interphase cytoplasm overexpressed eIF-1A. Nevertheless, compared with control nonmanipulated two-cell-stage embryos, reconstructed two-cell-stage embryos showed lower levels of expression of both eIF-1A and Gapdh, indicating a relatively low level of gene expression regardless the stage of the host cytoplast used.

DISCUSSION

Somatic nuclei that are transplanted into oocytes can be functionally reprogrammed to support embryonic development (Wilmut *et al.*, 1997; Kato *et al.*, 1998; Wakayama *et al.*, 1998). The molecular basis of reprogramming is unknown, and few differences in chromatin structure or composition between somatic and oocyte or early embryonic nuclei have been identified. Somatic histone H1 is immunologically undetectable in the nuclei of oocytes and one-cell embryos, whereas it is detectable in nuclei of older embryos (Clarke *et al.*, 1992, 1998; Smith *et al.*, 1995), and this may reflect functional differences between nuclei from these cell types. In this report, we have shown that following fusion of a blastomere of an eight-cell embryo to an oocyte, immunoreactive H1 is lost from the blastomere nucleus within 2–4 h of residence in the oocyte cytoplasm. Thus, with respect to this marker of chromatin configuration, blastomere nuclei exposed to oocyte cytoplasm be-

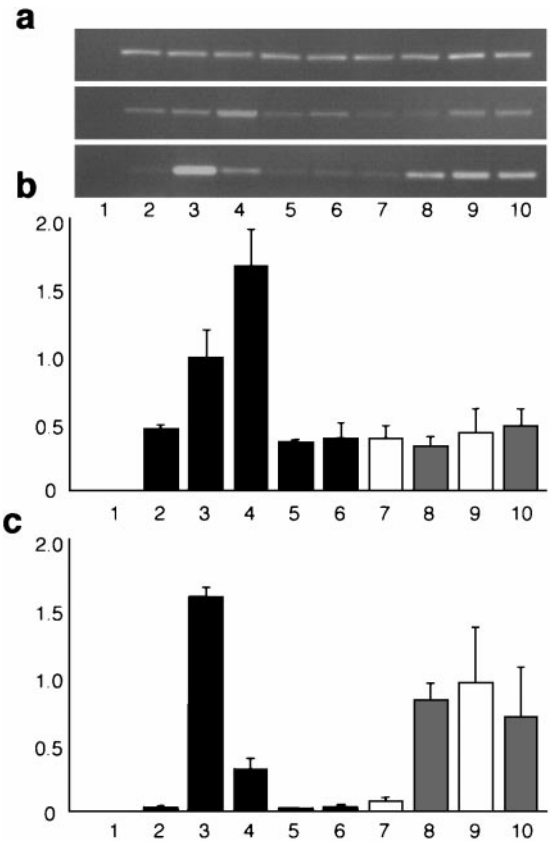

FIG. 5. Abundance of eIF-1a and Gapdh mRNA in embryos reconstructed using metaphase and interphase stage host oocytes. (a) Ethidium bromide-stained gel with the RT-PCR amplicons for α-globin (top), Gapdh (middle), and eIF-1A (bottom) photographs. Average densitometric quantification (bars) and standard errors (lines) of controls (black bars) and metaphase- (white bars) and interphase- (gray bars) reconstructed embryos for Gapdh (b) and eIF-1A (c) groups. Lanes represent a negative control (lane 1), metaphase II control (lane 2), two-cell control (lane 3), eight-cell control (lane 4), oocytes at 6 h (lane 5) and 16 h (lane 6) after activation, metaphase-(lane 7) and interphase- (lane 8) reconstructed one-cell stage embryos, and metaphase- (lane 9) and interphase- (lane 10) reconstructed two-cell-stage embryos.

TABLE 3

Loss of Immunoreactive H1 of Eight-Cell-Stage G1/S Nuclei Following Transfer into Bovine Ooplasts at Different Stages of the Cell Cycle

	Cell-cycle stage of recipient ooplast at time of fusion[a]								
	Metaphase II			Telophase II (3 h)			Interphase (8 h)		
Time fixed (h postfusion)	*n*	+	−	*n*	+	−	*N*	+	−
1	7	0	7	9	7	2	6	3	3
2	8	1	7	9	0	9	7	5	2
4	5	0	5	6	0	6	4	0	4
12	5	0	5	6	0	6	9	0	9

[a] (+) and (−), denote the presence and absence of somatic H1 staining of nuclei, respectively.

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come modified to resemble normal pronuclei. We previously observed a similar loss of immunoreactive H1 following nuclear transfer using bovine embryos (Bordignon *et al.,* 1999). Taken together, the results suggest that mammalian oocytes possess an activity that mediates the loss of the immunoreactive H1 epitope from chromatin.

Current evidence suggests that exposure of somatic nuclei to metaphase cytoplasm may enhance their ability to promote embryonic development (DiBerardino *et al.,* 1992; Wakayama *et al.,* 1998; Dominko *et al.,* 1999). In the present experiments, immunoreactive H1 was lost more rapidly when either donor nucleus or host ooplast were at metaphase than when either was at G1 or G2. Several factors may contribute to this. When the donor nucleus is at metaphase, the absence of a nuclear envelope may facilitate access to the chromatin of the activity mediating loss of immunoreactive H1. Similarly, when the host ooplast is at metaphase, even though the fusion procedure triggers parthenogenetic activation, the nuclear envelope of the donor nucleus may transiently break down or become permeabilized immediately following fusion (Barnes *et al.,* 1993; Collas *et al.,* 1992; Campbell *et al.,* 1993). This could allow rapid access of remodeling factors to the chromatin. It has previously been shown that experimental permeabilization of the nuclear envelope allows access to chromatin of factors that modulate chromatin activity (Blow and Laskey, 1988; Leno *et al.,* 1992).

A second possibility is that the phosphorylated H1 that would be present in metaphase chromosomes is more sensitive to the activity that mediates loss of immunoreactivity. Phosphorylation likely does not affect the affinity of H1 for the antibody, as in older embryos H1 is detectable at all cell cycle stages including metaphase (Clarke *et al.,* 1992; Smith *et al.,* 1995). Phosphorylation is reported to affect the stability of H1 in chromatin (Aubert *et al.,* 1991; Hill *et al.,* 1991), however, and thus could favor its interaction with remodeling factors or its displacement from chromatin. In this connection, the sperm-specific H1 becomes phosphorylated prior to its removal from sperm chromatin following fertilization in the sea urchin (Green and Poccia, 1985), whereas phosphorylation of H1 precedes but is not required for its removal from erythrocyte chromatin in *Xenopus* oocyte extracts (Dimitrov and Wolffe, 1996). In any case, the results indicate that cell-cycle conditions associated with effective functional reprogramming also are associated with rapid loss of immunoreactive H1.

We also found that immunoreactive H1 remained on nuclei transferred to hosts that were nonenucleated or were enucleated 8 h after activation when a well-formed pronucleus was present. By contrast, when only the chromosomes were removed from the hosts, so that soluble nuclear factors remained in the ooplast, immunoreactive H1 was lost. We propose that the loss of immunoreactive H1 requires factors that accumulate in the nucleus. According to this view, when the host nucleus remains in the oocyte, it acts as a sink for nuclear factors that are consequently unavailable to interact with the immunoreactive H1 on the donor nucleus.

The principle that oocyte nuclear factors accumulate in transplanted nuclei is well established. Somatic cell nuclei transferred into activated frog eggs undergo enormous swelling accompanied by the accumulation of labeled oocyte proteins within them (Merriam, 1969). Similarly, blastomere and somatic nuclei transplanted into mammalian eggs enlarge and modify their lamin composition, implying that they accumulate nucleophilic oocyte components (Stice and Robl, 1988; Szöllösi *et al.,* 1988; Prather *et al.,* 1990, 1991; Collas and Robl, 1991; Kubiak *et al.,* 1991; Stice *et al.,* 1994). Oocyte nuclear components are also required to remodel the sperm nucleus into the male pronucleus (Borsuk and Tarkowski, 1989). While molecular identity of most of these components is unknown, a major nucleoprotein in the frog oocyte is the histone chaperone, nucleoplasmin. The physiological role of nucleoplasmin is to promote exchange of sperm basic proteins for histones following fertilization (Philpott *et al.,* 1991; Philpott and Leno, 1992; Leno *et al.,* 1996). Interestingly, however, it also removes histone H1 from somatic nuclei transferred into oocyte cytoplasm (Dimitrov and Wolffe, 1996). Although nucleoplasmin has not been described in mammalian oocytes, it could be speculated that an analogous protein translocates into blastomere nuclei within ooplasm and mediates the loss of immunoreactive H1.

PKA activity is present in mouse embryos and is linked specifically to activation of the embryonic genome (Schwartz and Schultz, 1992). We observed that 6-DMAP or the protein kinase A-specific inhibitor, H-89, inhibited the loss of immunoreactive H1 from G2 nuclei but not from G1 nuclei. This suggests that G2 chromatin differs from that at G1 in some manner that is manifested by the G2-specific requirement for PKA activity to mediate the loss of immunoreactive H1. Differences in G1 and G2 nuclei have previously been identified. In *Xenopus* oocytes, for example, the minichromosome maintenance proteins that constitute part of the replication licensing factor are present on G1 chromatin but removed during S-phase progression (Tada *et al.,* 1999). In addition, the phosphorylation pattern of H1 varies during the cell cycle (Halmer and Gruss, 1996). One possibility is that, owing to the differences between G1 and G2 nuclei, a PKA-dependent cytoplasmic activity is required to remove immunoreactive H1 from G2 nuclei but not from G1 nuclei. Alternatively, PKA-dependent phosphorylation of H1 (Sweet *et al.,* 1997) or another chromatin component may be required for loss of immunoreactive H1 from G2 but not G1 nuclei.

We also observed that immunoreactive H1 was lost from mouse blastomere nuclei following transfer into bovine ooplasts. Moreover, in these interspecific transfers, this process occurred more rapidly in metaphase ooplasts than in telophase or interphase ooplasts, as it does in intraspecific transfers. These results suggest that the mechanism that is responsible for the loss of immunoreactive H1 is functionally conserved in diverse mammalian species. In-

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terestingly, it recently has been shown that nuclei of several mammalian species, when transferred to bovine ooplasts, are able to support development to the blastocyst stage (Dominko *et al.*, 1999). Our results indicate that this functional reprogramming in bovine ooplasm is accompanied by a specific molecular alteration in the foreign chromatin.

Finally, we observed that the expression of the developmentally regulated gene eIF-1A was abnormal in reconstructed embryos. Due to its transitory high expression at the two-cell stage, eIF-1A has been proposed to be involved in the process of embryonic gene activation (De Sousa *et al.*, 1998). We observed that the eIF-1A expression is markedly reduced after fusion to metaphase cytoplasm but remains high, and may be increased, after fusion to one-cell interphase cytoplasm. These results are in agreement with previous studies with bovine reconstructed embryos where metaphase cytoplasm induced a faster cessation of RNA synthesis compared to activated cytoplasm (Smith *et al.*, 1996). Moreover, the production of heterogeneous RNA in one-, two-, and four-cell stage embryos was higher after reconstruction with preactivated cytoplasm compared to control embryos (Lavoir *et al.*, 1997). In the mouse, the expression of the transcription-requiring complex genes was greater in embryos reconstructed with late than with early one-cell stage cytoplasm (Latham *et al.*, 1992). In contrast to the effect on eIF-1A, Gapdh expression was not affected in embryos reconstructed with either MII or interphase cytoplasm, suggesting that the reprogramming of donor chromatin may affect only the expression of specific genes. Indeed, as assessed by differential display analysis, less than 5% of mRNA bands differed between nuclear transfer and nonmanipulated control embryos (De Sousa *et al.*, 1999). Moreover, the expression of several specific genes was similar between reconstructed and control embryos (Winger *et al.*, 2000; Daniels *et al.*, 2000).

In conclusion, the collective results of numerous studies of nuclear-transfer embryos indicate that their developmental potential is influenced by the cell-cycle stage of both the donor nucleus and the host ooplast. While the optimal combination(s) remain to be determined, the results to date appear to indicate that embryonic development is better following transfer to metaphase cytoplasm than to interphase cytoplasm. Our results indicate that H1 removal is most rapid in metaphase oocytes and slowest or fails to occur in interphase cytoplasm and that it requires host nuclear factors that would be depleted from hosts that were enucleated after pronuclear formation, as well as phosphorylation in some cases. Thus, the cytoplasmic conditions that promote optimal development of nuclear-transfer embryos also promote rapid loss of immunoreactive H1 from the donor nuclei. As optimal development may require conditioning in the metaphase cytoplasm of unactivated oocytes, whereas there is rapid loss of immunoreactive H1 from metaphase oocytes that were activated at the time of fusion, it is likely that complete reprogramming requires other chromatin modifications. Nonetheless, our results suggest that loss of immunoreactive H1 is a useful molecular marker for, and may contribute to, the functional remodeling of somatic cell chromatin in ooplasm that produces a totipotential nucleus.

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